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Agrobacterium-mediated genetic transformation of *Camptotheca acuminata*

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Abstract: UGPase gene related with wood cellulose synthesis was transferred into *C. acuminata* using the method of *Agrobacterium*-mediated genetic transformation, and an efficient transformation system was developed for *C. acuminata* on the basis of evaluations of several factors affecting *Agrobacterium*-mediated DNA transfer rate. The highest transformation rate was achieved when pre-cultured leaf explants were infected with an *Agrobacterium* culture corresponding to OD600 (0.5) for 10 min, and cultured on explant regeneration medium for three days. The results of Southern hybridization showed that genomic DNA of the kanamycin-resistant shoots to an UGPase gene probe substantiated the integration of the transgene. Transformation efficiency (6%) was achieved under the optimized transformation procedure. This system should facilitate the introduction of important useful genes into *C. acuminata*.

Keywords: Genetic transformation; *Camptotheca acuminata*; UGpase gene

Introduction

Plant genetic transformation has become an important tool for functional genomics and is used as an adjunct to conventional breeding programs. Various methods and different approaches have been developed for the genetic transformation of plants. *Agrobacterium*-mediated gene transfer is one of the major techniques used for routine production of transgenetic plants. More than 100 species have been transformed by this method. It is perceived to have several advantages over other forms of transformation (such as biolistics), including the ability to transfer large segments of DNA with minimal rearrangement and with fewer copies of inserted genes at higher efficiencies cost (Hiei *et al*. 1997; Gheysen *et al*. 1998; Shibata *et al*. 2000)

Camptotheca acuminata Decaisne (Nyssaceae) has become one of major afforestation tree species in south China for its rapid growth. Its wood is good for paper making. At present, however, the pulpwood is very short for paper making in China. In the present study, the UGPase gene related with wood cellulose synthesis was transferred into *C. acuminata* for improving the cellulose content in its body, and some of the factors affecting the transformation efficiency and leading to the formation of transformed shoots were evaluated. To our knowledge, this is the first report for *Agrobacterium*-mediated genetic transformation of *C. acuminata*.

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Materials and methods

Plant material

C. acuminata leaves were collected from *in vitro* cultured plantlets. *In vitro* shoots were maintained by sub-culturing at 4-week intervals on B5 medium supplemented with 0.5-mg·L^{-1} BA, 20-g·L^{-1} sucrose and 7-g·L^{-1} agar. Isolated leaves for genetic transformation were removed leaf margins along with the tip and basal portions.

Plasmid and bacterial strains for transformation

The plasmid PBI121 containing the neomycin phosphotransferase II (nptII), UGPase genes were introduced into the *Agrobacterium* tumefaciens strains LBA4404 for transformation. This bacterial strain was maintained on a YEP (5-g·L^{-1} yeast extract, 5- g/L bactopeptone, 5-g·L^{-1} sucrose, pH 7.2) agar plate containing 50-mg·L^{-1} kanamycin and 100-mg·L^{-1} rifampicin.

Plant regeneration and transformation

A single colony was grown overnight in the dark in liquid YEP broth at 28°C with 50-mg·L^{-1} kanamycin and 100-mg·L^{-1} rifampicin (28°C, 180rpm). Bacterial cells of different OD at 600 nm were used for genetic transformation. Leaf explants, pre-cultured in shoot regeneration medium, were submerged in the bacterial suspension for varied time, blot dried on sterile paper and finally transferred to agar-solidified regeneration medium for different durations of co-cultivation. The explants were then transferred to shoot regeneration medium (0.7% agar-solidified) containing WPM salts added with 1.0-mg·L^{-1} BA, 0.2-mg·L^{-1} NAA and 30-g·L^{-1} sucrose, as well as 50-mg/L kanamycin and 500-mg·L^{-1} cefotaxine. Kanamycin-resistant shoots were sub-cultured at 15-day intervals. Media were adjusted to pH 5.8 prior to autoclaving at 121°C for 20 min. The cultures were incubated at 25 ± 2ºC, under a 16-h photoperiod of 40 μmol·m^{-2}·s^{-1} irradiance provided by white fluorescent tubes unless stated otherwise.

Evaluation of the factors affecting transformation

Three parameters were evaluated, including the length of pre-culture period (0, 1, 3, 5days), bacterial growth phase (OD600 values of 0.1, 0.2, 0.5, 0.8, 1.0), and length of the co-cultivation period (2, 3, 5 days). All of the parameters were evaluated and optimized on the basis of transformation efficiency. For each treatment, three replicates were used with a minimum of 30 explants per replicate. Means and standard errors were calculated by statistical analysis.

PCR analysis of transgenic plants

Total DNA was isolated from the leaves of untransformed and transformed plants using CTAB method of Doyle and Doyle (Doyle *et al*. 1990). PCR was performed in a volume of 20 μL containing 50 ng of genomic DNA. The primers employed were 35S 5'-acgcacaatcccactatccttcgc-3' and Nos 5'-tcgcaagaccggcaacaggattc-3'. The PCR program used had an initial strand separation step at 94°C for 30 s, annealing at 54°C for 1 min, elongation at 72°C for 2 min and a final extension step at 72°C for 10 min. The amplification products were separated by electrophoresis on 1% agarose gels. Molecular weights were estimated using DL2000 as a standard.

Southern blotting analysis

Total genomic DNA was extracted from the leaves of transgenic and non-transgenic control plants for Southern analysis using CTAB method. Total 20-μg DNA was digested overnight with BamHI to confirm the inserted UGPase gene in the genome. The digested DNA was separated by electrophoresis on 1% agarose gel. The 780bp PCR amplified product was labeled and used as hybridization probe. The whole southern blotting analysis was done according to the manufactures' instructions (Amersham ECL RPN3000).

Results and discussion

Effect of pre-culture period

The basis of the promotion of *Agrobacterium*-mediated transformation by pre-culturing explants has not been completely established, but it has been proposed that the production of vir-inducing compounds by metabolically active cells plays an important role (Spencer *et al*. 1991). It was reported that preculturing explants prior to inoculation with *Agrobacterium* improved the transformation frequency in several leguminous plant species (Singh *et al*. 2002; Husnain *et al*. 1997; Santarem *et al*. 1998; Pena *et al*. 1995), but decreased it in citrus (Costa *et al*. 2002). In our study, prior to infection with *Agrobacterium*, the leaf explants were cultured in the shoot regeneration medium during a period varying from 0 day to 5 days. Leaves pre-cultured for 3 days had the highest transformation efficiency (approx. 4.4%). No or shorter durations of pre-culture (one day) elicited a lower shoot organogenic response, while longer durations (5 days) resulted in more non-transformed shoots (Fig. 1).

Agrobacterium concentration

The number of *Agrobacterium* cells in the inoculum is considered to be a critical factor in the efficiency of transformation. An excessive number of bacteria can stress plant cells and affect their regeneration potential, whereas low concentrations can reduce the frequency of T-DNA transfer. For determining the right stage of *Agrobacterium* growth for high-efficiency transformation, five different growth phases were studied.

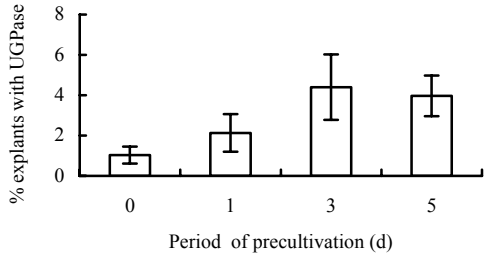

Fig. 1 Effects of length of pre-culture period on transformation efficiency of *C. acuminata*

Vertical bars represent the standard error of 30 explants

It is found that *Agrobacterium* concentration plays a significant role in transformation efficiencies. In the present study, *Agrobacterium* concentration at OD 0.1–0.5 were effective and the best result was obtained at OD 0.5 (Fig. 2). The results of our research showed that transformation frequency increased with *Agrobacterium* concentration at OD 0.1–0.5. It is likely that higher concentrations of *Agrobacterium* gave plant cells more chances to interact with *Agrobacterium*, consequently, more transformation events occurred. But further increase in *Agrobacterium* concentration from OD0.8 to OD1.0 proved to be detrimental, as the plant tissue died, which resulted in a very low number of transgenic plants.

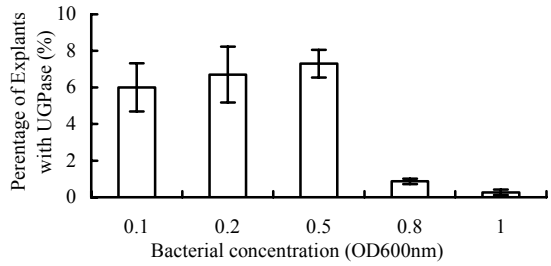

Fig. 2 Effect of concentration of *Agrobacterium* in the solution on transformation efficiency of *C. acuminata*

Vertical bars represent the standard error of 30 explants

Co-cultivation period

The duration of co-cultivation with *Agrobacterium* is one of the factors affecting transformation efficiency, and the optimal duration has been reported to be no longer than four days in many species (Cartis *et al*. 1999; Kondo *et al*. 2000). For determining the best duration of co-cultivation for *Agrobacterium*-mediated transformation of *C. acuminata*, transformation efficiency under co-cultivation periods of 2–5 d was investigated. The higher transformation efficiency was achieved after two and three days of co-cultivation (Fig. 3). The co-cultivation period of five days showed the lowest transformation efficiency due to the fact that the uncontrollable over-growth of bacteria resulted in explant necrosis, which led to a very low number of transgenic plants.

While Cervera *et al*. (1998) observed a higher transient transformation frequency of Carrizo citrange stem segments as the co-cultivation period increased, reaching a maximum at 5 days. Zhang *et al*. (2000) reported that in Chinese cabbage, the highest transformation frequency appeared in co-cultivation for 72 h. In general, most of studies show that higher transfer efficiencies occur at the co-culture time between two and three days (Muthukumar *et al*. 1996).

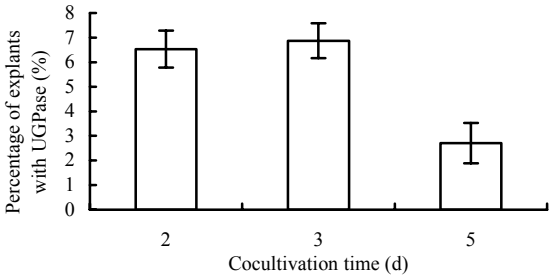

Fig. 3 Effects of length of co-cultivation period on transformation efficiency of *C. acuminate*

Vertical bars represent the standard error of 30 explants

Molecular analysis of transgenic *C. acuminata*

Integration of the T-DNA into the putative transgenic plant genomes was further confirmed by PCR and Southern blot analysis. An amplification of the expected 780-bp fragment corresponding to the UGPase gene was observed in all analyzed kanamycin-resistant shoots (Fig. 4, lane 4-7)， whereas, no amplification was detected in the samples from non-transformed plants (Fig. 4, lane 3). Although PCR analysis is a convenient method for the initial screening of putative transgenic plants, it does not prove the stable integration of transgenes, since detection by PCR could reflect the survival of *A. tumefaciens* in the host plant tissues. Therefore, southern blot analysis was conducted using the UGPase gene as a probe. Genomic DNA from putative transgenetic plants was digested with BamHI and allowed to hybridize with the UGPase.

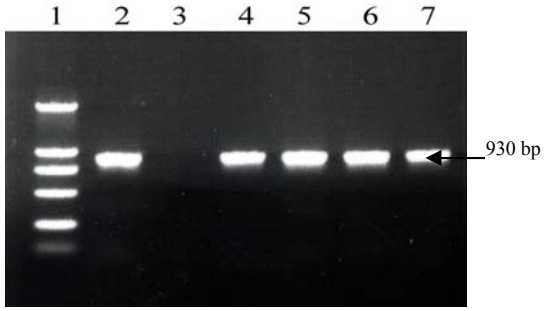

Fig. 4 Agarose gel electrophoresis of PCR-amplified fragments of the transgenes in transgenic *C. acuminata*

Lane 1, DL2000marker, Lane 2 plasmid as a positive control; Lane 3, non-transformed control plant; Lane 4-7 transgenic plant

Southern analysis of five randomly chosen plants (both kanamycin resistant and PCR-positive plantlets) showed that four of them confirmed the presence and integration of the UGPase fragment gene into the *C. acuminata* (Fig. 5, lane 3-6). No hybridization occurred with DNA isolated from control plants (non-transformed, lane 1).

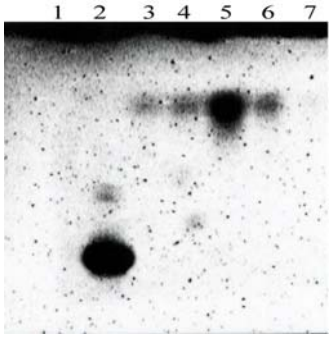

Fig. 5 Southern blot analysis of the integrated UGPase gene in *C. acuminate*

BamHI digestion of genomic DNA from transgenic plants (Lanes 3-7) generated transgene fragment that hybridized to the UGPase probe, while the non-transformed control (lane 1) had no such fragment. Lane 2 UGPase plasmid DNA generated transgene fragment that hybridized to the UGPase probe.

In conclusion, an efficient system for the production of transgenic *C. acuminata* was established via *Agrobacterium*-mediated transformation. The transformation system developed in this study will facilitate *C. acuminata* genetic improvement via genetic engineering.

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